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Biosynthesis of the Methanogenic Cofactors

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Our current knowledge of the pathways and genes involved in the biosynthesis of the methanogenic coenzymes methanopterin, coenzyme B, methanofuran, coenzyme F_{420}, and coenzyme M is presented. Proposed reaction mechanisms for several of the novel reactions involved in the pathways are presented. © 2001 Academic Press.

I. Introduction

This review on the biosynthesis of the methanogenic cofactors in the methanoarchaea will constitute an update of published and unpublished work on this topic since it was last reviewed in 1993 (White and Zhou, 1993). The structures of the cofactors are shown in the figures and include methanopterin, coenzyme B, methanofuran, coenzyme F_{420}, and coenzyme M. These coenzymes are referred to as the methanogenic coenzymes, because each functions as a catalyst in the biochemical reduction of CO_2 or acetate to CH_4 (DiMarco *et al.*, 1990).

The present status of work on each of the coenzymes will be presented using the figures, which show the biosynthetic pathways, as the focus of the discussion. Most of the work reported involves the cloning and overexpression of *Methanococcus jannaschii* gene products. Because they are derived from the smallest hyperthermophilic autotrophic methanogenic archaeal genome currently sequenced, and therefore fewer genes have to be considered, identification of the desired genes is more likely. We have also confirmed that *M. jannaschii* contains the coenzymes that are discussed in this chapter (Graupner and White,

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2000). Furthermore, purification of the overexpressed enzymes in *Escherichia coli* is easily accomplished since most of the *E. coli* proteins precipitate on heating, whereas the *M. jannaschii* proteins—being from a hyperthermophile—do not precipitate. Measurements of the biosynthetic reactions of the overexpressed enzymes have been performed with extracts of *Methanosarcina thermophila*, because these cells consistently show higher activities than those observed with other methanoarchaea.

II. Biosynthesis of Methanopterin

Methanopterin (MPT), in its tetrahydro reduced state, functions as a C_1 carrier coenzyme in methanogenesis (DiMarco *et al.*, 1990; Weiss and Thauer, 1993; Thauer, 1998) in a capacity analogous to that seen in folate biochemistry, where ^5N-formyltetrahydrofolate is converted to ^5N-^{10}N-methylene-tetrahydrofolate and finally to ^5N-methyltetrahydrofolate (Matthews, 1996). On the baisis of chemical and stereochemical (White, 1996b) similarities between the structures of tetrahydrofolate and tetrahydromethanopterin, some common connections between the biosynthesis of these two cofactors were considered likely. These connections have been confirmed by a series of early labeling experiments, which show that GTP (**I**) is the precursor of the pterin and that 4-aminobenzoic acid is the precursor of the arylamine portion of the molecule (White and Zhou, 1993).

Of the seventeen reactions involved in the biosynthesis of the reduced form of methanopterin (**XXI**) (Fig. 1), all reactions but the one analogous to the dihydrofolate reductase, reaction 17, have been demonstrated to occur in cell extracts of *M. thermophila* (White, 1990, 1996a), an organism containing sarcinapterin, an analog of methanopterin. The steps in the biosynthesis of MPT in *Methanobacterium thermoautotrophicum* strains ΔH and Marburg have been shown to be essentially the same as those established for *M. thermophila*, demonstrating the universal occurrence of this pathway in the methanogens (White, 1998).

So far, only three genes in the Methanopterin biosynthetic pathway (Fig. 1) have been identified in *Methanococcus jannaschii:* MJ0798, MJ0301, and MJ1425. The enzyme derived from gene MJ0798 catalyzes reaction 1 in Fig. 1; that from MJ0301, reaction 7; and that from MJ1425, reaction 18. Each of these genes has been confirmed by the overexpression of the gene product and by establishing that it catalyzes the desired reaction.

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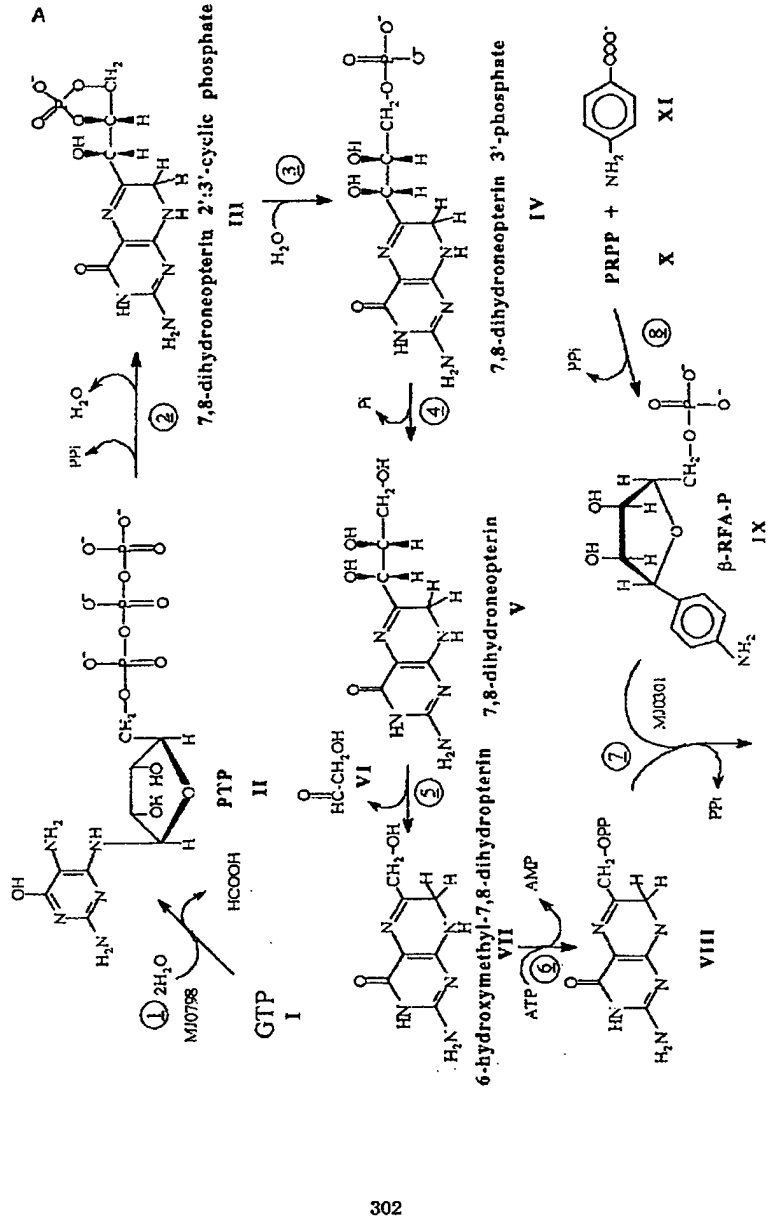
The biosynthetic pathway to MPT begins with the removal of the C-8 carbon of GTP (I) to form triaminopyrimidine triphosphate (PTP, II), a previously unidentified natural product. The removal of the C-8 carbon of GTP is a common feature of both the GTP cyclohydrolase I reaction, involved in neopterin biosynthesis (Green *et al.,* 1996), and the GTP cyclohydrolase II reaction, involved in riboflavin biosynthesis (Bacher *et al.,* 1996) in both bacteria and eukaryotic organisms. The reactions required for the archaeal transformation are consistent with the first two mechanistic steps involved in the bacterial GTP cyclohydrolase II reaction (Foor and Brown, 1975) but are not consistent with the GTP cyclohydrolase I mechanism (Nar *et al.,* 1995). The formation of compound II has been shown in our laboratory to be catalyzed by the enzyme encoded by the *M. jannaschii* gene MJ0798. Compound II is not an intermediate in the GTP cyclohydrolase I or II enzymatic reactions, but it is an intermediate in methanopterin biosynthesis in *M. thermophila* (Howell and White, 1997). Compound II would, however, appear to serve as a common precursor for methanopterin, riboflavin, and coenzyme F_{420} in the methanoarchaea (see later). The methanoarchaeal GTP cyclohydrolase may in fact be related to GTP cyclohydrolase II, the first enzyme in bacterial riboflavin biosynthesis, since it has been identified by a 21-amino acid sequence with 25% identity to the *Escherichia coli* GTP cyclohydrolase II. The enzyme also has an 81-

FIG. 1. Pathway and genes involved in the biosynthesis of 5,6,7,8-tetrahydromethanopterin (XXI). In all the figures included in this review, intermediates are indicated by roman numerals, reactions by underlined numbers, and reactions that have been confirmed in cell extracts and/or with the cloned gene products by circled underlined reaction numbers. Reactions for which the genes have been assigned are indicated by the *M. jannaschii* gene number or the gene name, if this has been assigned. The gene numbers refer to genes assigned in *M. jannaschii* (Bult *et al.,* 1997). **I,** Guanosine triphosphate; **II,** triaminopyrimidine triphosphate; **III,** 7,8-dihydroneopterin 2′:3′-cyclic phosphate; **IV,** 7,8-dihydroneopterin 3′-phosphate; **V,** 7,8-dihydroneopterin; **VI,** glycolaldehyde; **VII,** 6-hydroxymethyl-7,8-dihydropterin; **VIII,** 6-hydroxymethyl-7,8-dihydropterin pyro-phosphate; **IX,** 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate; **X,** 5-phospho-β-D-ribosyl-1-pyrophosphate; **XI,** 4-aminobenzoic acid; **XII,** 7,8-dihydropterin-6-ylmethyl-4-(β-D-ribofuranosyl) aminobenzene 5′-phosphate; **XIII,** 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol 5′-phosphate; **XIV,** 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol; **XV,** 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5′-phosphate]-D-ribitol; **XVI,** 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5′-diphosphate]-D-ribitol; **XVII,** 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5′-triphosphate]-D-ribitol; **XXII,** α-ketoglutarate; **XXIII,** (*S*)-2-hydroxyglutaric acid; **XVIII,** didemethylated 7,8-dihydromethanopterin; **XIX,** monomethylated 7,8-dihydrometh-anopterin; **XX,** 7,8-dihydromethanopterin; **XXI,** 5,6,7,8-tetrahydromethanopterin.


A
GTP
I
MJ0798
2H2O
HCOOH
PTP
II
7,8-dihydroneopterin 2':3'-cyclic phosphate
III
PPi
H2O
H2O
6-hydroxymethyl-7,8-dihydropterin
VII
7,8-dihydroneopterin
V
HC-CH2OH
VI
7,8-dihydroneopterin 3'-phosphate
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IX
PRPP + NH2
X
XI

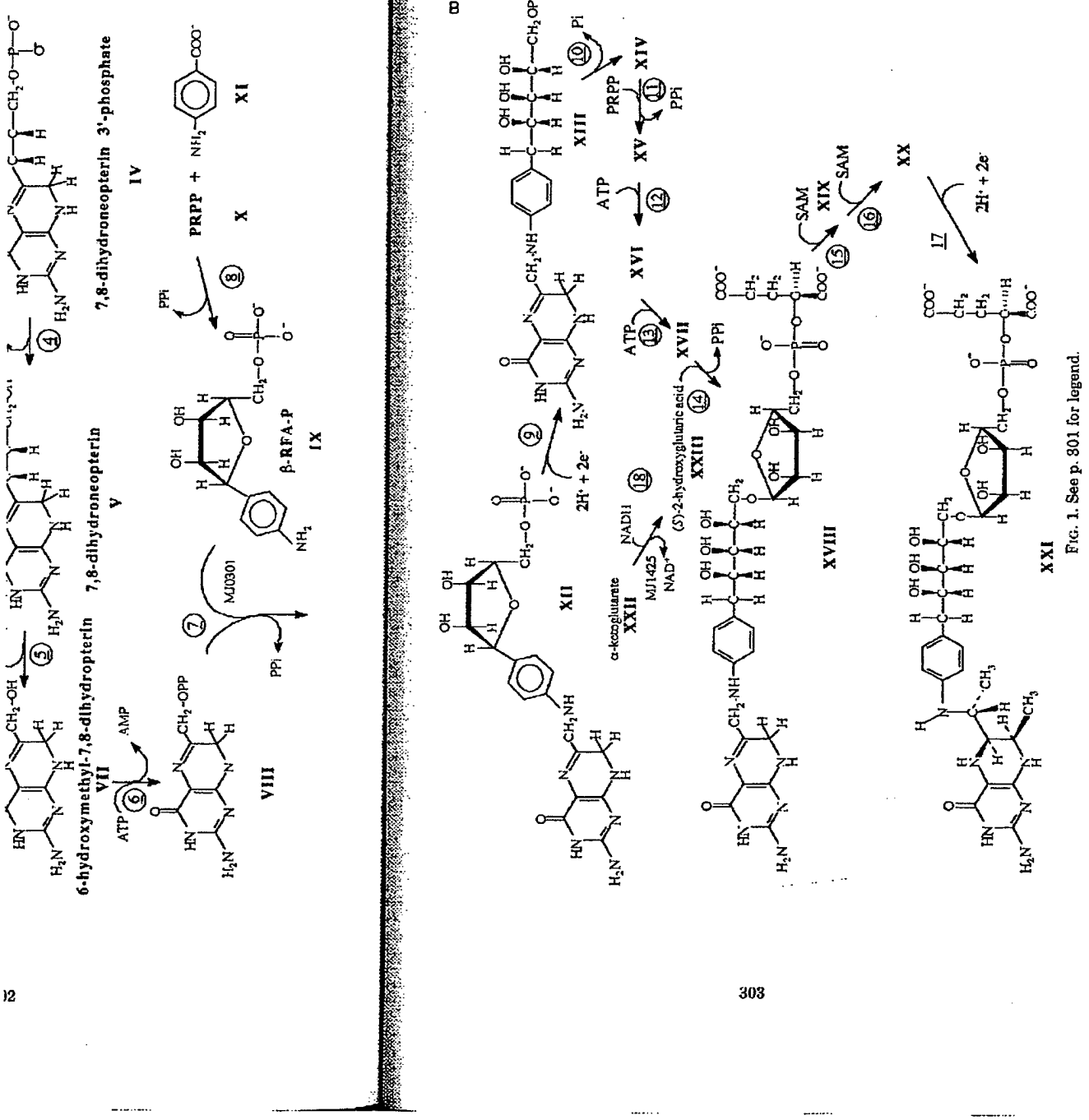

Fig. 1. See p. 301 for legend.

amino acid sequence having 21% identity with the putatively identified *Archaeoglobus fulgidus* GTP cyclohydrolase II derived from AF0484 (Klenk *et al.*, 1998). We have named the *M. jannaschii* enzyme "GTP cyclohydrolase III" since the product of its enzymatic reaction, compound **II**, is different from that produced by either GTP cyclohydrolase I, which produces 7,8-dihydroneopterin triphosphate, or GTP cyclohydrolase II, which produces triaminopyrimidine monophosphate (**I** in Fig. 9) (Howell and White, 1997).

Compound **II** is then converted to 7,8-dihydroneopterin 2':3'-cyclic phosphate (**III**) (Fig. 1, reaction 2), an intermediate identified by incubating a cell extract of *M. thermophila* with GTP (Howell and White, 1997). A proposed mechanism for this reaction is shown in fig. 2. Hydrolysis of the cyclic phosphate in compound **III** (reaction 3 in Fig. 1) leads to 7,8-dihydroneopterin 3'-phosphate (**IV**), which is then hydrolyzed to 7,8-dihydroneopterin (**V**) by an unknown phosphatase (reaction 4 in Fig. 1).

The next two reactions in MPT biosynthesis (reactions 5 and 6 in Fig. 1) are identical to the reactions in folate biosynthesis and would be expected to be catalyzed by dihydroneopterin aldolase and by 6-hydroxymethyldihydropterin pyrophosphokinase, respectively. For both of these reactions, the bacterial genes are known (Lopez and Lacks, 1993; Talarico *et al.*, 1992), and the X-ray crystal structure for the enzyme catalyzing both reactions 5 and 6 has been reported (Hennig *et al.*, 1998, 1999; Xiao *et al.*, 1999; Stammers *et al.*, 1999). Despite this information, none of the genes for these enzymes can be found in the genomes of the archaea *M. jannaschii, M. thermoautotrophicum* strain ΔH, or *A. fulgidus,* based on sequence comparisons. It would appear that either nonorthologous enzymes are responsible for catalyzing these reactions in the methanoarchaea, or the sequences of the enzymes have diversified to such an extent that they cannot be detected by sequence comparisons.

The next reaction in the pathway (reaction 7 in Fig. 1) is the condensation of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (**VIII**) with 4-(β-D-ribofuranosyl)aminobenzene 5'-phosphate (β-RFA-P, **IX**) to form **XII.** This reaction is mechanistically analogous to the 7,8-dihydropteroate synthase in bacterial folate biosynthesis (Green *et al.*, 1996), but it utilizes **IX** in place of 4-aminobenzoic acid (**XI**) or *N*-(*p*-aminobenzoyl)-L-glutamic acid. Again, no gene with sequence homology to the 7,8-dihydropteroate synthase can be readily identified in the archaeal genomes. The gene encoding the enzyme has been identified however, using a novel computer program, ORF (an acronym for Ostensible Recognition of Folds), developed by Aurora and Rose (1998) for

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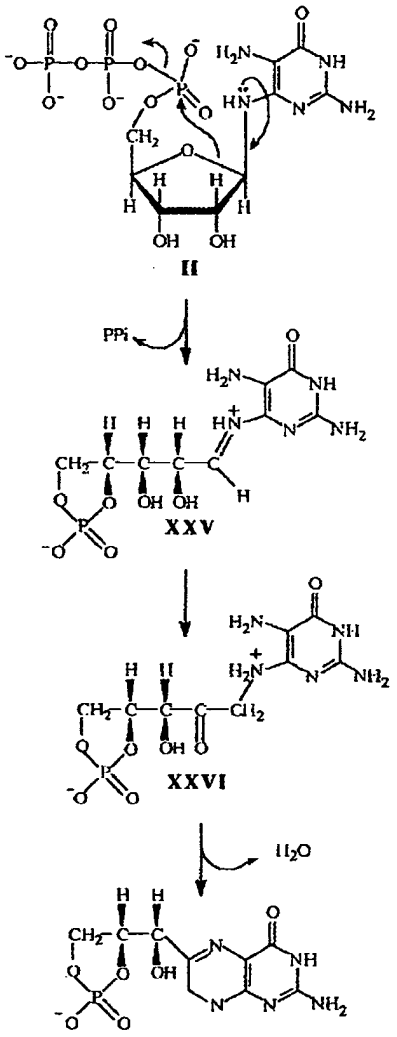

7,8-dihydroneopterin 2':3'-cyclic phosphate
III

FIG. 2. Proposed intermediates in the conversion of triaminopyrimidine triphosphate (II) to 7,8-dihydroneopterin 2':3'-cyclic phosphate (III). II, Triaminopyrimidine triphosphate; III, 7,8-dihydroneopterin 2':3'-cyclic phosphate; XXV and XXVI are proposed intermediates in the pathway.

the identification of genes in genomic databases. This program can identify open reading frames with low sequence homologies based on their predicted secondary structure distributions when compared with secondary structure distributions of a known enzyme. The enzyme identified in this search, derived from *M. jannaschii* gene MJ0301, has only 19.9% sequence similarity to the fungal 7,8-dihydropteroate synthase from *Pneumocystis carinii*. The enzyme has been overexpressed and shown to catalyze reaction 7 (Fig. 1; Xu *et al.*, 1999). The enzyme does not catalyze the formation of 7,8-dihydropteroate from **XI** and **VIII**. This is also found to be true for gene product of MJ0107, which has been putatively assigned as a 7,8-dihydropteroate synthase (Bult *et al.*, 1997). The discovery that the *M. jannaschii* thymidylate synthase, derived from MJ0757, uses methylenetetrahydrofolate as the cofactor for the formation of dTMP (Xu *et al.*, 1999) is surprising because the methanoarchaea are considered to lack folates (Leigh, 1983; Worrell and Nagle, 1988).

One of the unique steps in the MPT biosynthetic pathway (reaction 8 in Fig. 1) is the condensation of **XI** with 5-phospho-α-D-ribosyl diphosphate (PRPP, **X**) to produce **IX**. This reaction, catalyzed by the *M. thermophila* β-RFA-P synthase, is unique among known phosphoribosyltransferases (PRTases) in that a decarboxylation of one of the substrates (**XI**) occurs during the reaction, and a *C*-riboside rather than a *N*-riboside is formed at the site of the decarboxylation (Rasche and White, 1998). The enzymatic reaction is very specific for **XI**, with 4-hydroxybenzoic acid serving as the only alternate substrate. This reaction is, however, analogous to other PRTase reactions in that pyrophosphate is released as a product of the reaction and the reaction proceeds with a net inversion of sterochemistry at C-1 of the ribose, as shown in Fig. 3. Evidence for this mechanism was obtained by the $NaCNBH_3$ reduction of intermediate **I** to β-D-ribofuranosylbenzene 5′-phosphate (Rasche and White, 1998).

Compound **XII** is reduced to compound **XIII** (reaction 9 in Fig. 1) in a reaction stimulated by the addition of FMN or coenzyme F_{420} to the reaction mixture (White, 1996a); as with reaction 10, no information is available concerning the enzymes or genes for these reactions. A possi-

FIG. 3. Mechanisms for reactions 8 and 11 (see Fig. 1) in methanopterin biosynthesis. **IX**, 4-(β-D-Ribofuranosyl)aminobenzene 5′-phosphate; **X**, 5-phospho-β-D-ribosyl-1-pyrophosphate; **XI**, 4-aminobenzoic acid; **XXVII**, oxonium ion formed by the loss of pyrophosphate from PRPP; **XIV**, 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol; **XV**, 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5′-phosphate]-D-ribitol; **XXVIII**, intermediate I.

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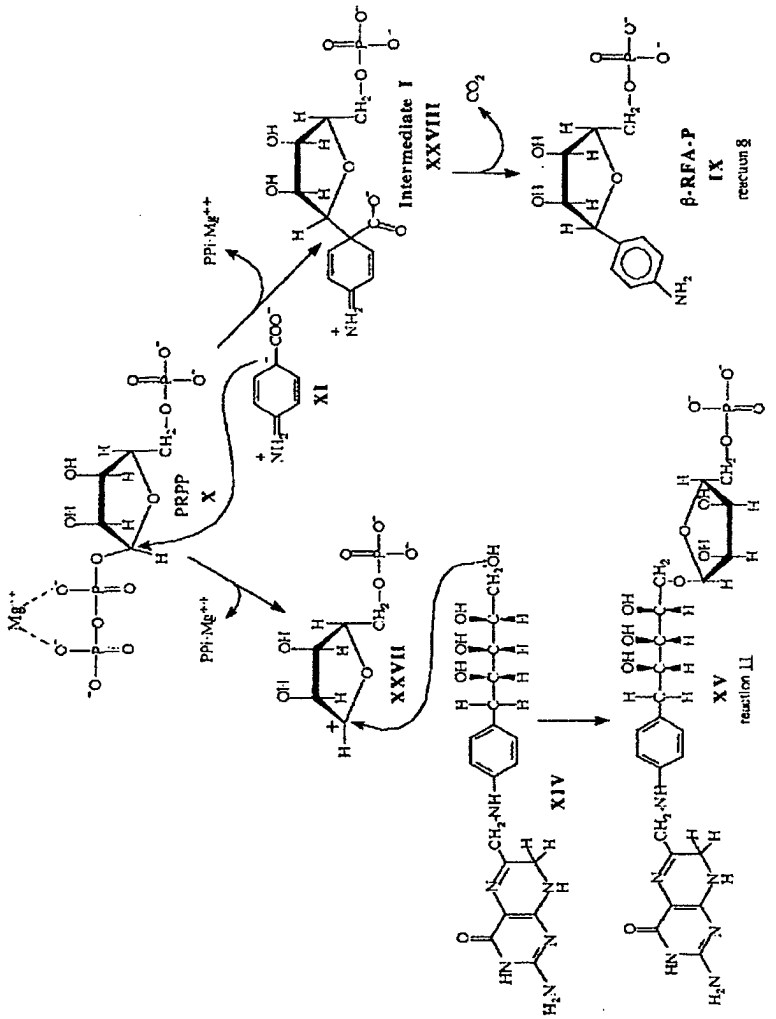

(see Fig. 1) in methanopterin biosynthesis. phosphate; **X**, 5-phospho-β-D-ribosyl-1-py- **II**, oxonium ion formed by the loss of py- erin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy- l-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl e I.

ble mechanism for this reaction would involve the intramolecular elimination of an alcohol in **XII** to form a substituted methylene quinone imine (**XXIX**) that would then be reduced to compound **XIII** (Fig. 4).

Reaction 11 in Fig. 3 is another unique biochemical reaction for a PRTase since the product of the reaction, **XV** (**XIII** with an α-linked ribose phosphate in place of the terminal phosphate), contains an α-linked ribosyl group. All previous known examples of PRTase products

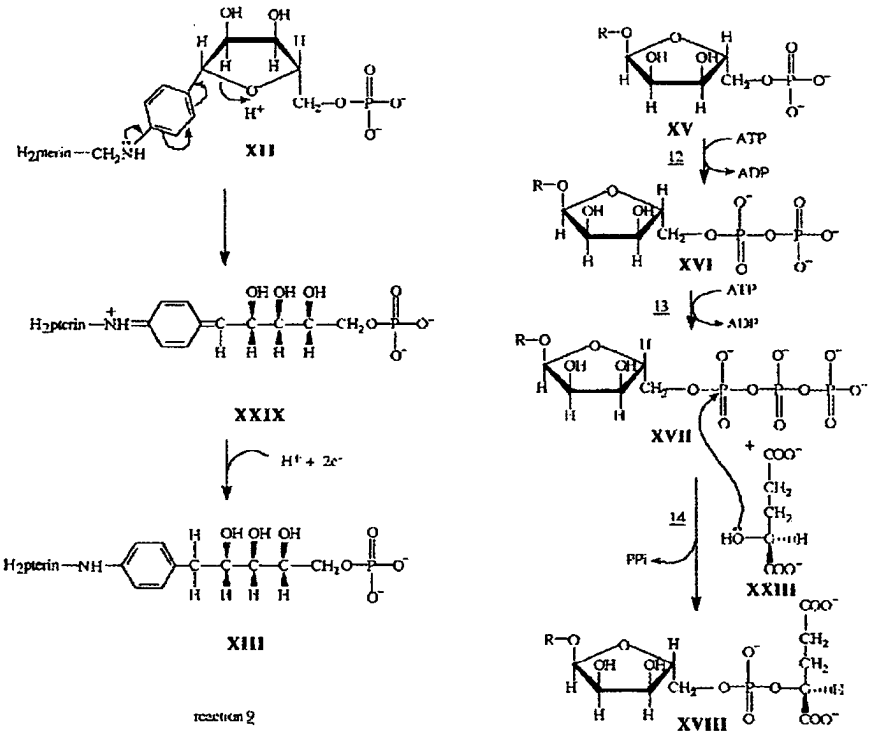

FIG. 4. Mechanisms for reactions 9, 12, 13, and 14 (see Fig. 1) in methanopterin biosynthesis. **XII**, 7,8-Dihydropterin-6-ylmethyl-4-(β-D-ribofuranosyl)aminobenzene 5'-phosphate; **XIII**, 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-D-ribitol 5'-phosphate; **XV**, 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofurenosyl 5'-phosphate]-D-ribitol; **XVI**, 7,8-dihydropterin-6-ylmethyl-1-(4- aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5'-diphosphate]-D-ribitol; **XVII**, 7,8-dihydropterin-6-ylmethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ribofuranosyl 5'-triphosphate]-D-ribitol; **XXIII**, (*S*)-2-hydroxyglutaric acid; **XVIII**, didemethylated 7,8-dihydromethanopterin.

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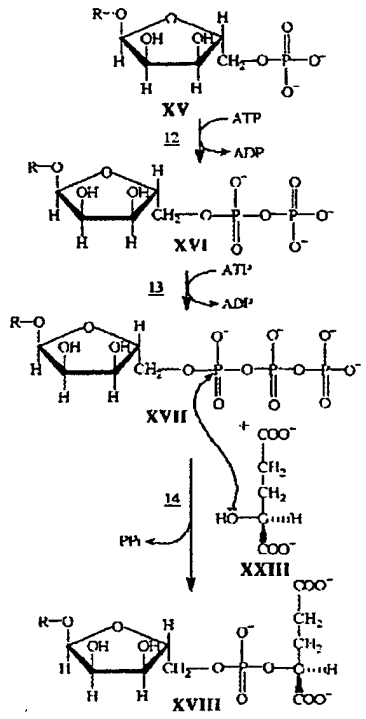

13, and 14 (see Fig. 1) in methanopterin
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methyl-1-(4-aminophenyl)-1-deoxy-D-ribitol
ethyl-1-(4-aminophenyl)-1-deoxy-5-[1-α-D-ri-
7,8-dihydropterin-6-ylmethyl-1-(4- amino-
liphosphate]-D-ribitol; **XVII**, 7,8-dihydrop-
-5-[1-α-D-ribofuranosyl 5′-triphosphate]-D-
II, didemethylated 7,8-dihydromethanopterin.

are β-ribosides (Musick, 1981). Considering the expected mechanisms of these reactions, which likely involve as a first step the generation of a C-1 oxonium ion on the ribose as shown in Fig. 3 (Murry *et al.*, 1997), it is easy to see how an enzyme could evolve from an existing PRTase by simply allowing the nucleophilic portion of the substrate—in our case the primary hydroxyl group—to approach from the α side. If this is the case, then the enzyme could have easily evolved from a member of the known PRTase family of enzymes.

Although the exact mechanism for the conversion of **XV** to **XVIII** is not presently known, considering the chemistry involved one would assume that **XV** is converted to **XVI** with an enzyme possibly related to a nucleoside monophosphate kinase. The resulting **XVI** would then be further phosphorylated to **XVII** by an enzyme related to a nucleoside diphosphate kinase. Finally, displacement of pyrophosphate with the hydroxyl group of **XXIII** would lead to **XVIII**. This proposed mechanism, shown in Fig. 4, is of course the same as that used by nature in the biosynthesis of the phosphodiesters in nucleic acids and further confirms an early metabolic connection between the biosynthesis of coenzymes and that of nucleic acids (White, 1976).

Labeling experiments with cell extracts of *M. thermoautotrophicum* strain ΔH and Marburg and *M. thermophila* strain TM-1 have established that the sites in the overall sequence of reactions from **II** to 5,6,7,8-tetrahydromethanopterin (**XXI**)—where the *S*-adenosylmethionine (SAM)-dependent C-9 and C-7 methylations of the pterin-containing intermediates occur—are dependent on the species of methanoarchaea (White, 1998). I have proposed that MJ0563 and/or MJ1200, putatively identified as DNA-cytosine methyltransferases, may be the genes encoding the enzymes responsible for these methylation reactions (reactions 15 and 16 in Fig. 1) (White, 1998). This idea is supported by the observation that these methanoarchaea do not contain 5-methylcytosine-modified bases (J. A. McClosky, personal communication, 1996). Since the methylations are sensitive to thiol reagents, a mechanism requiring the addition of the thiol to the different enamine isomers of the dihydropterin, as shown in Fig. 5, has been proposed (White, 1998). This reaction mechanism is analogous to that proposed for thymidylate synthase (Carreras and Santi, 1995) and 2′-deoxyuridylate hydroxymethylase (Kunitani and Santi, 1980). It has also been demonstrated during this work that cells of *M. thermautotrophicum* strains ΔH and Marburg contain significant amounts of methanopterin that lacks the phosphate and 2-hydroxyglutaric acid groups (White, 1998).

Given that the absolute stereochemistry of the hydroxyglutaric acid

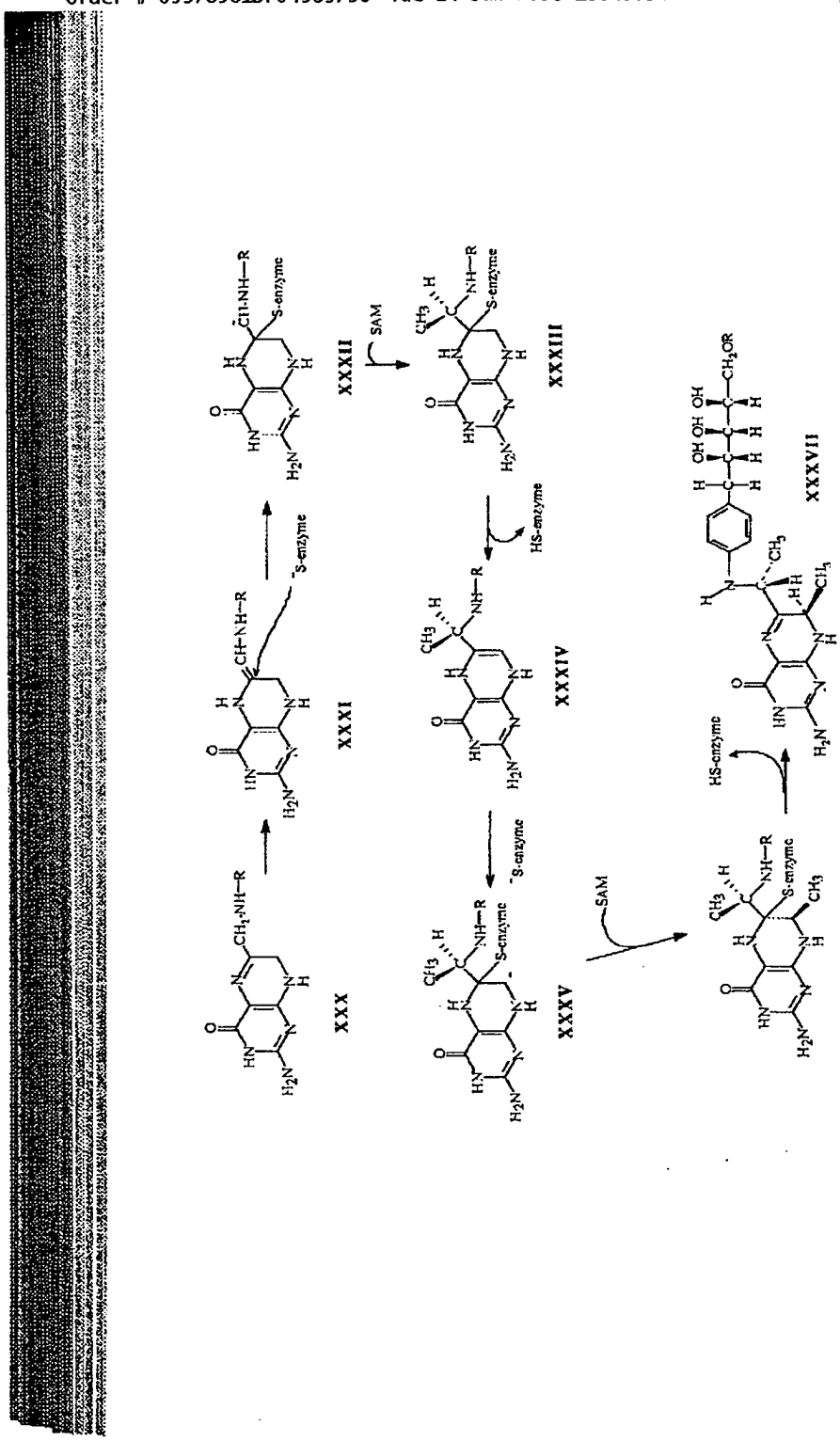


FIG. 6. Proposed mechanism for the methylation of the intermediates leading to methanopterin. The chemical structure(s) of the specific methanopterin intermediates that are methylated is species dependent. Thus the R group can represent several different structures.

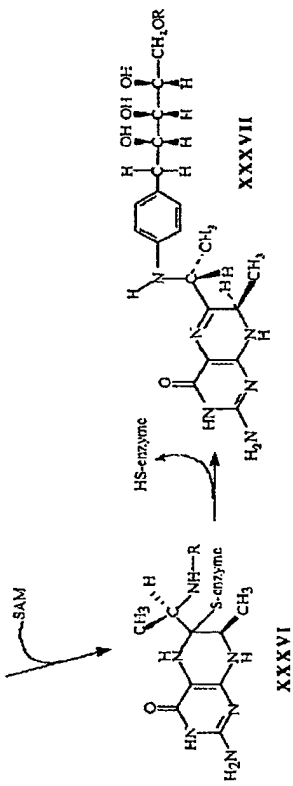


FIG. 5. Proposed mechanism for the methylation of the intermediates leading to methanopterin. The chemical structure(s) of the specific methanopterin intermediates that are methylated is species dependent. Thus the R group can represent several different structures.

present in MPT has been established as (*S*)-hydroxyglutaric acid (**XXIII**) (Solow and White, 1997a), it is considered likely that this moiety of MPT is produced by the reduction of α-ketoglutaric acid by a lactate/malate dehydrogenase-like enzyme. The *M. jannaschii* genome contains two genes predicted to produce homologs of the lactate/malate dehydrogenase group of enzymes, MJ0490 and MJ1425. We have cloned and overexpressed the enzyme derived from each of these genes and have determined that the protein derived from MJ1425 functions as an α-ketoglutarate reductase, producing **XXIII** by the NADH-dependent reduction of α-ketoglutarate (**XXII**) (Graupner *et al.*, 2000). Thus, this enzyme is very likely involved in the production of the **XXIII** moiety of the side chain of methanopterin (reaction 18 in Fig. 1). This same enzyme also reduces sulfopyruvate to L-sulfolactate and thus functions in the biosynthesis of coenzyme M (see later).

Many additional analogs of methanopterin function throughout the archaea (White, 1993a,b, 1997), and methanopterin has now been found in organisms outside of the archaeal domain (Chistoserdova *et al.*, 1998; Pomper *et al.*, 1999). Since the structures of all of these modified folates have common features, they will surely be found to have related biosynthetic pathways.

III. Biosynthesis of Coenzyme B

The biochemistry of the steps involved in the conversion of α-ketoglutarate and acetyl coenzyme A (acetyl-CoA) to α-ketosuberate, a precursor of coenzyme B [7-mercaptoheptanoylthreonine phosphate (**CVII**)] and of biotin, in *Methanosarcina thermophila*, and its further conversion to coenzyme B, is shown in Fig. 6. Most of the enzymes or genes involved in the pathway are now established and are indicated in Fig. 6. The biosynthesis of the alkyl portion of coenzyme B and biotin in the archaea is an interesting dilemma, considering that these cells contain no fatty acids (Kates, 1993) and have no genes homologous to the fatty acid synthases (Bult *et al.*, 1997).

Several years ago, a pathway for the formation of the 7-mercaptoheptanoic acid (**CV**) moiety of coenzyme B (**CVII**) was proposed based on ^{13}C-labeling studies and the identification of α-ketoglutarate (**XXII**), α-ketoadipate (**XCV**), α-ketopimelate (**XCIX**), and α-ketosuberate (**CIII**) in a series of methanoarchaea (White, 1989a,b). In the proposed pathway the repeated application of the α-ketoacid chain elongation series of reactions was used to increase the number of methylenes from two, as found in **XXII**, to five, as found in the product, **CIII**. Compound **CIII**

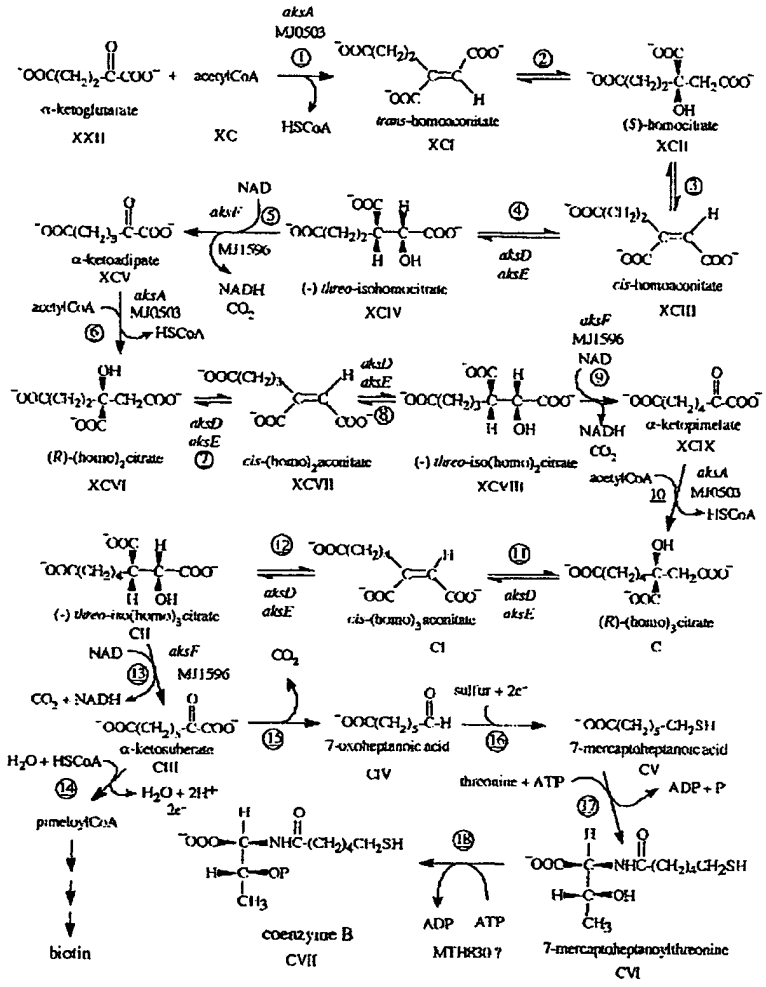
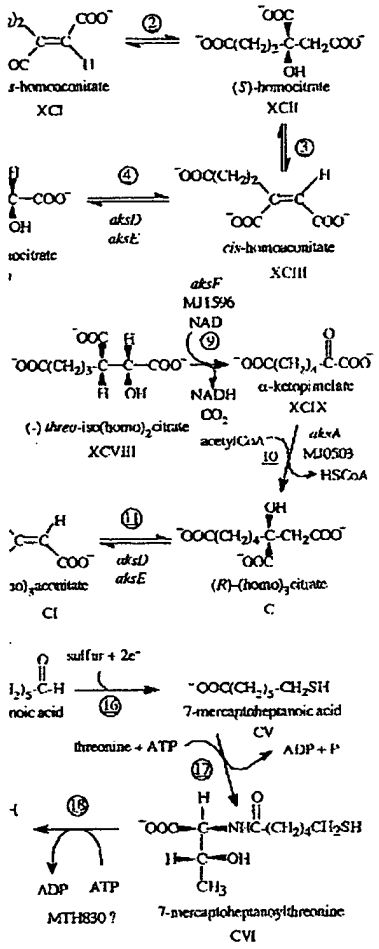


FIG. 6. Pathway and genes involved in the biosynthesis of coenzyme B and biotin. **XXII,** α-Ketoglutarate, 2-oxopentanedioic acid; **XC,** acetyl coenzyme A; **XCI,** *trans*-homoaconitate, (*E*)-1,2,4-but-1-enetricarboxylic acid; **XCII,** (*S*)-homocitrate, (*S*)-2-hydroxy-1,2,4-butanetricarboxylic acid; **XCIII,** *cis*-homoaconitate, (*Z*)-1,2,4-but-1-enetricarboxylic acid; **XCIV,** (−)-*threo*-isohomocitrate, (2*R*,3*S*)-1-hydroxy-1,2,4-butanetricarboxylic acid; **XCV,** α-ketoadipate, 2-oxohexanedioic acid; **XCVI,** (*R*)-(homo)$_2$citrate, (*R*)-2-hydroxy-1,2,5-pentanetricarboxylic acid; **XCVII,** *cis*-(homo)$_2$aconitate, (*Z*)-1,2,5-pent-1-enetricarboxylic acid; **XCVIII,** (−)-*threo*-iso(homo)$_2$citrate, (2*R*,3*S*)-1-hydroxy-1,2,5-pentanetricarboxylic



iosynthesis of coenzyme B and biotin. **XXII**, acetyl coenzyme A; **XCI**, *trans*-homoaconi- **II**, (*S*)-homocitrate, (*S*)-2-hydroxy-1,2,4-bu- tate, (*Z*)-1,2,4-but-1-enetricarboxylic acid; droxy-1,2,4-butanetricarboxylic acid; **XCV**, **I**, (*R*)-(homo)$_2$citrate, (*R*)-2-hydroxy-1,2,5- aconitate, (*Z*)-1,2,5-pent-1-enetricarboxylic ‹,3*S*)-1-hydroxy-1,2,5-pentanetricarboxylic

then serves as a precursor to the biosynthesis of the 7-mercaptoheptanoic acid (**CV**) moiety of coenzyme B (White, 1989c). The process thereby represents another route to the biosynthesis of fatty acid-like compounds from acetate, adding only the methyl carbon of acetate with each cycle of the process.

Oddly, these series of reactions have been found to begin with the condensation of **XXII** and acetyl-CoA (**XC**) to form *trans*-homoaconitate (**XCI**) (Fig. 6, reaction 1). The resulting compound **XCI** is then hydrated and dehydrated to **XCIII**, with (*S*)-homocitrate (**XCII**) serving as an intermediate (reactions 2 and 3 in Fig. 6). The formation of **XCI** has been shown to be catalyzed by the protein product of the *M. jannaschii* gene MJ0503, which is homologous to homocitrate synthases. The unusual aspect of this reaction is that the product of the reaction is not the expected (*R*)-homocitrate but **XCI** produced by the dehydration of the expected product homocitrate. Considering the homology between this enzyme and homocitrate synthase, we must conclude that the enzyme catalyzes not only the condensation between **XXII** and **XC** but also a dehydration reaction, generating the observed product **XCI**. Oddly, this same enzyme has been observed to catalyze the formation of (*R*)-(homo)$_2$citrate (**XCVI**) and (*R*)-(homo)$_3$citrate (**C**) from their respective reactants without the dehydration step occurring (Howell *et al.*, 1998). It would appear that the enzyme has evolved to specifically catalyze the required series of elongation reactions shown in Fig. 6.

The discovery of the presence of both **XCII** and **XCI** in the cell extracts of *M. thermophila*, as well as their production in the incubation mixtures, is unexpected since neither of these compounds has ever been previously reported as a natural product. Their discovery complicates the expected simplicity of the formation of (−)-*threo*-isohomocitrate from α-ketoglutarate and acetyl-CoA, a process expected to occur via *cis*-homoaconitate (**XCIII**) and (*R*)-homocitrate, as in the biosynthesis of lysine via the α-ketoadipate pathway (Rodwell, 1969).

Knowing that the precursor to the α-ketoadipate (**XCV**) is **XCIV**, the same isohomocitrate isomer that is involved in lysine biosynthesis, and knowing that only trans additions and eliminations of water are involved in the interconversions of the intermediates (Gawron and Ma-

acid; **XCIX**, α-ketopimelate, 2-oxoheptanedioic acid; **C**, (*R*)-(homo)$_3$citrate, 2-hydroxy-1,2,6-hexanetricarboxylic acid; **CI**, *cis*-(homo)$_3$aconitate, (*Z*)-1,2,6-hex-1-enetricarboxylic acid; **CII**, (−)-*threo*-iso(homo)$_3$citrate, (2*R*,3*S*)-1-hydroxy-1,2,6-hexanetricarboxylic acid; **CIII**, α-ketosuberate, 2-oxooctanedioic acid; **CIV**, 7-oxoheptanoic acid; **CV**, 7-mercaptoheptanoic acid; **CVI**, 7-mercaptoheptanoylthreonine; **CVII**, coenzyme B, 7-mercaptoheptanoylthreonine phosphate.

hajan, 1966), the reaction sequences 2, 3, and 4 shown in Fig. 6 have been proposed to account for the first set of reactions in the pathway. This pathway accounts for the formation of all of the intermediates observed. Reactions 2 and 3 would not be expected to be carried out by an enzyme homologous to aconitase or homoaconitase, since these enzymes use only **XCIII** and (*R*)-homocitrate as substrates (Beinert *et al.*, 1996). This idea has been strengthened by demonstrating that the two subunit aconitases formed by the different combinations of the proteins encoded by *M. jannaschii* genes MJ1003, MJ1271, MJ0499, and MJ1277 have not been found to catalyze reactions 2 and 3. Reaction 4, however, is that expected for an aconitase or a homoaconitase-like enzyme and is catalyzed by mixtures of these enzymes. It is possible that reaction 3 in Fig. 6 is catalyzed by the aconitate hydrase described by Neilson (1956a,b). Unfortunately, this enzyme appears to have never been purified nor isolated in a pure form; therefore, no information is currently available as to the stereospecificity of its reaction mechanism. Reaction 2 in Fig. 6 could be catalyzed by an enzyme related to mesaconase, an enzyme that catalyzes the reversible addition of water to mesaconate (*trans*-1,2-prop-1-enedicarboxylic acid) to form (*S*)-citramalate (Flint and Allen, 1996; Wang and Barker, 1969). The direct conversion of **XCI** to **XCIII** with an enzyme analogous to aconitate isomerase is not possible on mechanistic grounds (Klinman and Rose, 1971).

Rehydration of **XCIII** produces (−)-*threo*-isohomocitrate (**XCIV**), which undergoes a NAD-dependent oxidative decarboxylation to produce **XCV**. This reaction, which is analogous to an isocitrate dehydrogenase reaction, has been shown to be catalyzed by the protein product of the *M. jannaschii* gene MJ1596 (Howell *et al.*, 2000b). This gene product, now designated as AksF, and its homolog MJ0940—both originally assigned as isocitrate dehydrogenase (Bult *et al.*, 1997)—in fact do not catalyze the isocitrate dehydrogenase reaction with isocitrate as a substrate. The **XCV** resulting from this reaction then undergoes two consecutive sets of α-ketoacid chain elongation reactions to produce **CIII**. In each of these sets of reactions, it has been shown that the homologs of *cis*-homoaconitate, homocitrate, and (−)-*threo*-isohomocitrate serve as intermediates. The gene product of MJ0503 also catalyzes the condensation of **XCV** or **XCIX** with acetyl-CoA to form the (*R*)-homocitrate homologs of (*R*)-2-hydroxy-1,2,5-pentanetricarboxylic acid and of (*R*)-2-hydroxy-1,2,6-hexanetricarboxylic acid, respectively. The aconitase-like enzyme formed from the protein product of MJ1003 and MJ1271, designated AksDE, catalyzes reactions 4, 7, 8, 11, and 12 (see Fig. 6).

Compound **CIII** resulting from this series of reactions then undergoes a nonoxidative decarboxylation to form 7-oxoheptanoic acid (**CIV**)

s 2, 3, and 4 shown in Fig. 6 have st set of reactions in the pathway. ıtion of all of the intermediates ob- be expected to be carried out by an r homoaconitase, since these en- ıitrate as substrates (Beinert *et al.*, ıed by demonstrating that the two 'erent combinations of the proteins)03, MJ1271, MJ0499, and MJ1277 tions 2 and 3. Reaction 4, however, homoaconitase-like enzyme and is ıes. It is possible that reaction 3 in te hydrase described by Neilson ıe appears to have never been pu- erefore, no information is current- y of its reaction mechanism. Reac- an enzyme related to mesaconase, ıle addition of water to mesaconate l) to form (*S*)-citramalate (Flint and). The direct conversion of **XCI** to aconitate isomerase is not possible d Rose, 1971).

(−)-*threo*-isohomocitrate (**XCIV**), oxidative decarboxylation to pro- ıalogous to an isocitrate dehydro- e catalyzed by the protein product owell *et al.*, 2000b). This gene prod- homolog MJ0940—both original- ase (Bult *et al.*, 1997)—in fact do ınase reaction with isocitrate as a this reaction then undergoes two ɔngation reactions to produce **CIII.** ıs been shown that the homologs of (−)-*threo*-isohomocitrate serve as J0503 also catalyzes the condensa- A to form the (*R*)-homocitrate ho- ɛtricarboxylic acid and of (*R*)-2-hy- respectively. The aconitase-like uct of MJ1003 and MJ1271, desig- , 8, 11, and 12 (see Fig. 6).

is series of reactions then under- to form 7-oxoheptanoic acid (**CIV**)

(reaction 15), a precursor to coenzyme B, and an oxidative decarboxylation to form pimeloyl-CoA (reaction 14), the precursor to biotin. The 7-oxo group of the 7-oxoheptanoic acid is then converted to a thiol by a previously described route (reaction 16) (White, 1989c). The details of the mechanism of this reaction are not presently understood, but they involve a protein-bound sulfur, derived from H_2S, as the source of the sulfur. The resulting 7-mercaptoheptanoic acid (**CV**) is converted, by an ATP-dependent process, to 7-mercaptoheptanoyl-L-threonine (**CVI**) (reaction 17) (Solow and White, 1997b). The current evidence indicates that this reaction proceeds by the formation of 7-mercaptoheptanoyl phosphate by the transfer of the γ-phosphate of the ATP to **CV.** Reaction of the amino group of L-threonine with the 7-mercaptoheptanoyl phosphate produces **CVI.** ATP-dependent phosphorylation of the **CVI** produces the final coenzyme B (reaction 18 in Fig. 6) (White, 1994). Growing cells of both *Methanococcus volta* and *M. thermophila* have been found to readily incorporate **CV** and **CVI** into coenzyme B. In the case of **CVI** the incorporation proceeds without cleavage of the amide bond (White, 1994).

IV. Biosynthesis of Methanofuran

The most recent investigation relating to the biosynthesis of methanofuran has concentrated on the biosynthesis of the 1,3,4,6-hexanetetracarboxylic acid (HTCA) portion of methanofuran (**LXXXIII**). A summary of the present status of this work is shown in Fig. 7 and is in agreement with earlier labeling studies (Eisenreich and Bacher, 1992; Gorkovenko *et al.*, 1994; White, 1987). All of the circled reactions shown in the pathway have been confirmed by the synthesis of each intermediate and by the demonstration that each is converted to one or more of the indicated intermediates, using cell-free preparations of *M. thermophila* (Howell *et al.*, 2000a). The pathway proposed for the biosynthesis of HTCA emerges in a logical manner from the intermediates detected in the methanogens, from the intermediates shown to be metabolized by cell extracts of *M. thermophila,* and from known information about biochemical reactions. The unusual aspect of the pathway is the cooccurrence of two different stereoisomers of the same compound functioning in a parallel manner in many of the steps in the pathway.

The first step in the pathway is the synthesis of *trans*-homoaconitate (**XCI**) from **XXII** and **XC** under the direction of the *aksA* gene product AksA (Fig. 7, reaction 1). This step is also the first step in the biosynthesis of α-ketosuberate, a precursor to the 7-mercaptoheptanoyl moi-

ety of coenzyme B, as discussed earlier in this chapter. Compound **XCI** thus represents a branch point metabolite leading to both coenzyme B and methanofuran. Trans addition of water to **XCI** produces an *erythro*-isohomocitrate (**LX**) (Fig. 7, reaction 2). This reaction involves the same trans addition that occurs with aconitase and fumarase enzymes, but it proceeds with a trans substrate analogous to that observed with the enzyme mesaconase (Flint and Allen, 1996; Wang and Barker, 1969). Although there is currently no protein sequence information available for any mesaconase, the genomes of *M. jannaschii* (Bult *et al.*, 1997), *M. thermoautotrophicum* ΔH (Smith *et al.*, 1997), and *A. fulgidus* (Klenk *et al.*, 1998) each have two sets of genes (Discussed in Section III) homologous to an aconitase or isopropylmalate dehydratase, with one member of each set containing the required [4Fe–4S] binding sites. We have cloned and overexpressed the enzymes from each of these *M. jannaschii* genes and have demonstrated that no combination of any of these proteins catalyzes any of the dehydration reactions involved in HTCA biosynthesis. The enzyme catalyzing this reaction is, however, not unique to the archaea since *E. coli* cell extracts have also been shown to readily catalyze this reaction (R. H. White, unpublished results, 1998). At present it has not been possible to establish which of the enantiomers of *erythro*-isohomocitrate is used in this pathway, since no method has been devised for the synthesis or isolation of the different enantiomers of **LX**. However, this is the first report of the occurrence and metabolism of an *erythro* isomer of isohomocitrate in nature.

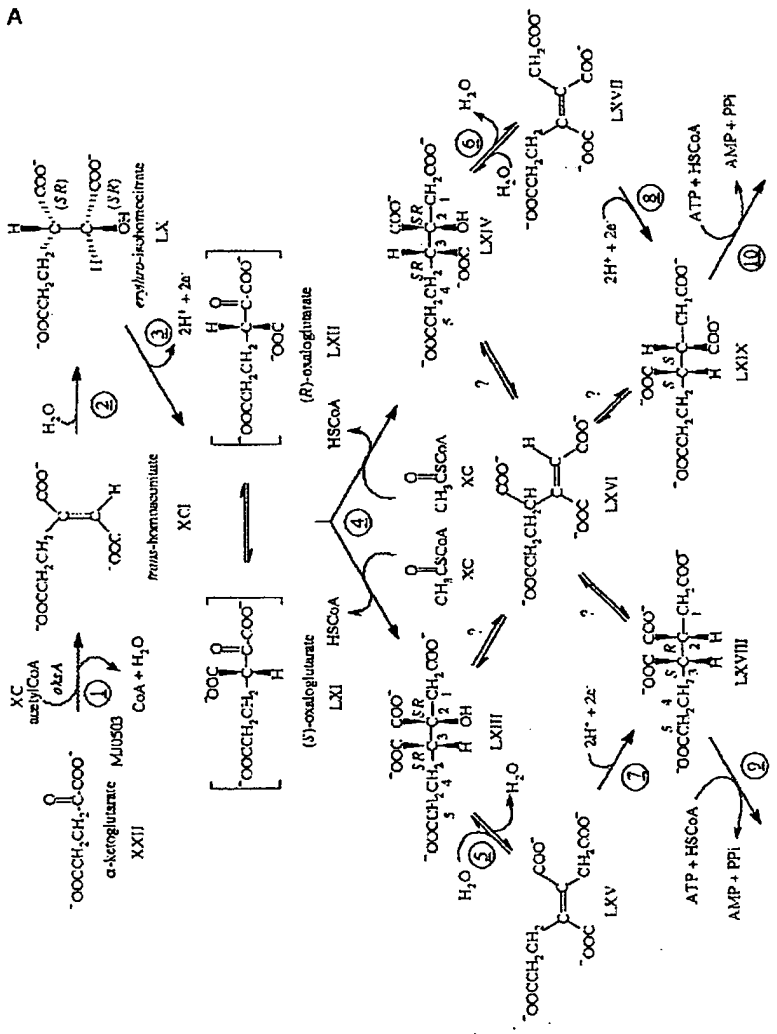
The conversion of **LX** to the two different diastereomers of 1-hydroxy-1,3,4,5-pentanetetracarboxylic acid, **LXIII** and **LXIV** (Fig. 7, reaction 4), can be viewed as taking place in two separate steps. The first step consists of the oxidation of the hydroxyl group of **LX** to a keto group to produce oxaloglutarate, which then condenses with **XC** to form **LXIII** and **LXIV** in the second step. These reactions would be analogous to those used by the combined action of malate dehydrogenase (EC 1.1.1.37) and citrate synthase (EC 4.1.3.7). So far, it has not been possible to show that oxaloglutarate is an intermediate in this reaction. We therefore propose that the oxaloglutarate is a protein-bound intermediate as occurs in the isocitrate dehydrogenase-catalyzed reactions (Siebert *et al.*, 1957). An unusual aspect of the *erythro*-isohomocitrate reaction is that at least two different isomers are produced in the incubation, **LXIII** and **LXIV**. The production of these two isomers could be the result of two separate enzymes, one using (*S*)-oxaloglutarate (**LXI**) and the other using (*R*)-oxaloglutarate (**LXII**), or, more likely, it results by a single enzyme that is not sensitive to the stereochemistry at C-3 of **LXI** or **LXII** when carrying out reaction 4. It is interesting to note

r in this chapter. Compound **XCI** olite leading to both coenzyme B vater to **XCI** produces an *erythro*-). This reaction involves the same tase and fumarase enzymes, but ılogous to that observed with the 1996; Wang and Barker, 1969). ı sequence information available *jannaschii* (Bult *et al.*, 1997), *M. l.*, 1997), and *A. fulgidus* (Klenk ıes (Discussed in Section III) ho-ylmalate dehydratase, with one uired [4Fe–4S] binding sites. We zymes from each of these *M. jan-*d that no combination of any of ehydration reactions involved in lyzing this reaction is, however, *oli* cell extracts have also been n (R. H. White, unpublished re-ın possible to establish which of ate is used in this pathway, since nthesis or isolation of the differ-s is the first report of the occur-mer of isohomocitrate in nature. rent diastereomers of 1-hydroxy-**XIII** and **LXIV** (Fig. 7, reaction vo separate steps. The first step yl group of **LX** to a keto group to ndenses with **XC** to form **LXIII** reactions would be analogous to of malate dehydrogenase (EC .3.7). So far, it has not been pos-intermediate in this reaction. We rate is a protein-bound interme-ydrogenase-catalyzed reactions ect of the *erythro*-isohomocitrate somers are produced in the incu-on of these two isomers could be e using (*S*)-oxaloglutarate (**LXI**) (**LXII**), or, more likely, it results ve to the stereochemistry at C-3 ıction 4. It is interesting to note

that the C-3 hydrogen of oxaloglutarate is expected to be acidic, leading to rapid racemization at this carbon. This fact could account for the mixture of reaction products observed. The production of **LXIII** and **LXIV** now generates a branch point in the biosynthetic pathway where these two different isomers are metabolized to the final HTCA product.

The conversion of **LXIII** to (*E*)-1,3,4,5-pent-2-enetetracarboxylic acid (**LXV**) and that of **LXIV** to (*Z*)-1,3,4,5-pent-2-enetetracarboxylic acid (**LXVII**) are simple dehydration reactions. Each reaction could be catalyzed by an enzyme with a mechanism analogous to that observed in either an aconitase- or a fumarase-type reaction and would be expected to proceed by the trans elimination of water. The production of (*Z*)-1,3,4,5-pent-1-enetetracarboxylic acid (**LXVI**) is also observed in these experiments and could arise by the trans elimination of water from the 1,2 positions of either **LXIII** or **LXIV**. Although most fumarases are relatively specific for fumarate, hydration of a series of substituted fumarates has been observed by Eck and Simon (1994b), and a related enzyme could be involved in the proposed pathway. The dehydration reaction could have been carried out by one of the aconitases present in *M. jannaschii* discussed earlier, but this has not been found to be the case.

FIG. 7. Pathway for the biosynthesis of the 1,3,4,6-hexanetetracarboxylic acid moiety of methanofuran. **XXII**, α-Ketoglutarate; **XCI**, *trans*-homoaconitate, (*E*)-1,2,4-but-1-enetricarboxylic acid; **LX**, *erythro*-isohomocitrate, (1*SR*,2*SR*)-1-hydroxy-1,2,4-butanetricarboxylic acid; **LXI**, (*S*)-oxaloglutarate, (*S*)-1-oxo-1,2,4-butanetricarboxylic acid; **LXII**, (*R*)-oxaloglutarate, (*R*)-1-oxo-1,2,4-butanetricarboxylic acid; **LXIII**, *erythro*-HPTCA, (2*SR*,3*SR*)-2-hydroxy-1,2,3,5-pentanetetracarboxylic acid; **LXIV**, *threo*-HPTCA, (2*SR*, 3*RS*)-2-hydroxy-1,2,3,5-pentanetetracarboxylic acid; **LXV**, (*E*)-2-PETCA, (*E*)-1,2,3,5-pent-2-enetetracarboxylic acid; **LXVI**, (*Z*)-1-PETCA, (*Z*)-1,2,3,5-pent-1-enetetracarboxylic acid; **LXVII**, (*Z*)-2-PETCA, (*Z*)-1,2,3,5-pent-2-enetetracarboxylic acid; **LXVIII**, L-*erythro*-PTCA, L-*erythro*-(2*R*,3*S*)-1,2,3,5-pentanetetracarboxylic acid; **LXIX**, L-*threo*-PTCA, L-*threo*-(2*S*,3*S*)-1,2,3,5-pentanetetracarboxylic acid; **LXX**, coenzyme A ester of L-*erythro*-(2*R*,3*S*)-1,2,3,5-pentanetetracarboxylic acid; **LXXI**, L-*xylo*-HHTCA, (1*S*,3*S*,4*S*)-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid; **LXXII**, coenzyme A ester of L-*threo*-(2*S*,3*S*)-1,2,3,5-pentanetetracarboxylic acid; **LXXIII**, L-*erythro*-KHTCA, (3*R*,4*S*)-1-oxo-1, 3,4,6-hexanetetracarboxylic acid; **LXXIV**, L-*threo*-KHTCA, (3*S*,4*S*)-1-oxo-1,3,4,6-hexanetetracarboxylic acid; **LXXV**, L-*arabino*-HHTCA, (1*R*,3*R*,4*S*)-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid; **LXXVI**, L-*ribo*-HHTCA, (1*S*,3*R*,4*S*)-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid; **LXXVII**, L-*lyxo*-HHTCA, (1*R*,3*S*,4*S*)-1-hydroxy-1,3,4, 6-hexanetetracarboxylic acid; **LXXVIII**, coenzyme A ester of (1*R*,3*R*,4*S*)-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid; **LXXIX**, coenzyme A ester of (1*S*,3*R*,4*S*)-1-hydroxy-1,3,4, 6-hexanetetracarboxylic acid; **LXXX**, L-*erythro*-(*E*)-1,3,4,6-hex-1-enetetracarboxylic acid, (3*R*,4*S*)-HETCA; **LXXXI**, L-*threo*-(*E*)-1–1,3,4,6-hex-1-enetetracarboxylic acid, (3*S*,4*S*)-HETCA; **LXXXII**, coenzyme A ester of HTCA, (2*RS*,3*SR*)-1,3,4,6-hexanetetracarboxylic acid or *meso*-HTCA; **LXXXIII**, methanofuran.


A
XC
acetylCoA
citA
MJ0503
CoA + H2O
α-ketoglutarate
XXII
trans-homoaconitate
XCI
erythro-isohomocitrate
LX
2H+ + 2e-
(R)-oxalosuccinate
(S)-oxalosuccinate
LXI
HSCoA
LXII
XC
LXIII
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AMP + PPi
H2O
2H+ + 2e-

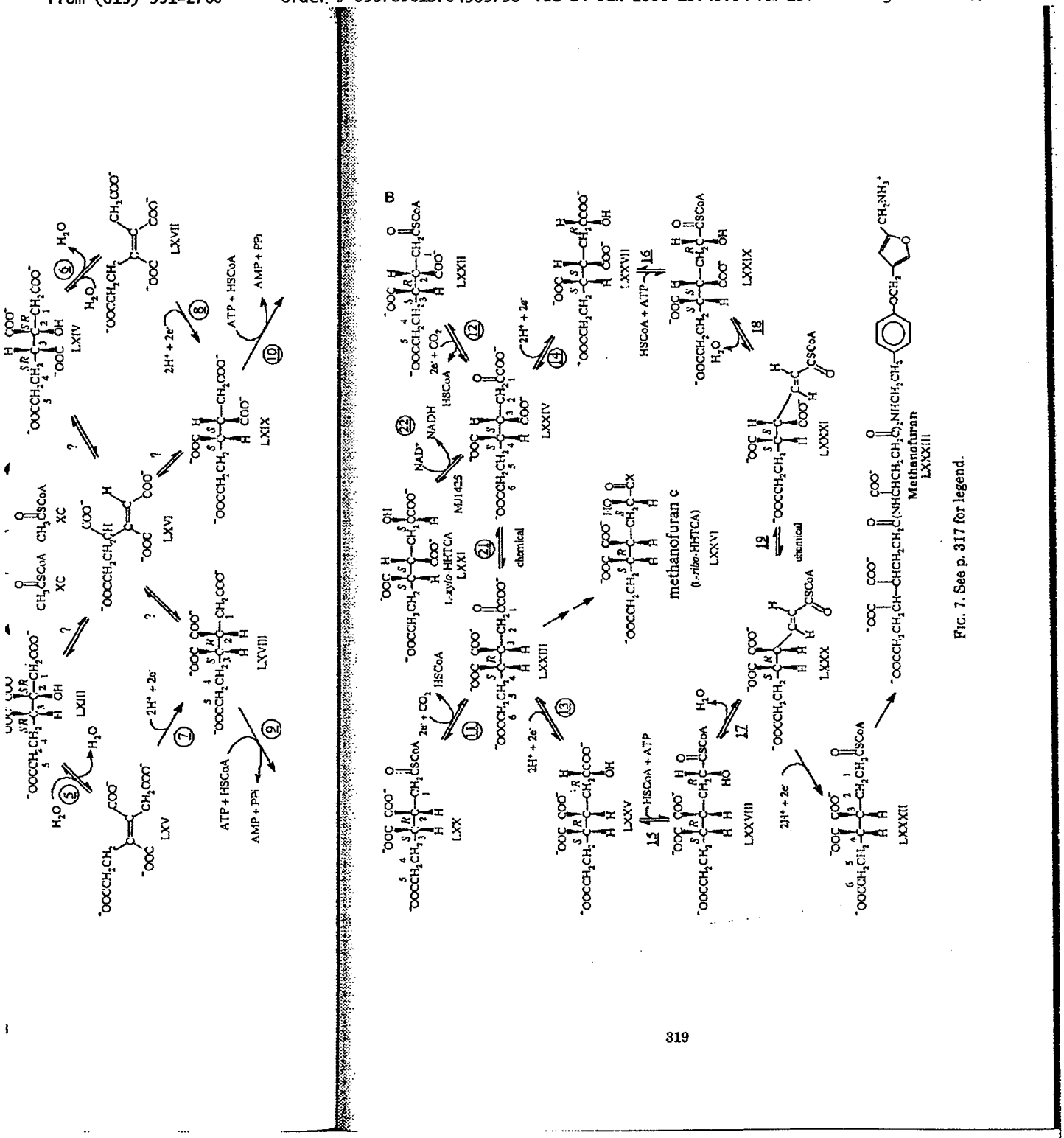

FIG. 7. See p. 317 for legend.

The hydrogenation of **LXV** to L-*erythro*-1,3,4,5-pentanetetracarboxylic acid (**LXVIII**) and of **LXVII** to L-*threo*-1,3,4,5-pentanetetracarboxylic acid (**LXIX**) (Fig. 7, reactions 7 and 8) would both be expected to proceed by a trans hydrogenation, as observed in the reduction of fumarate (Tchen and van Milligan, 1960), substituted fumarates (Eck and Simon, 1994a), and all known examples of the biochemical reduction of monounsaturated acids (White, 1980). The archaeal reductions (reactions 7 and 8) are most analogous to those carried out by either fumarate reductase or succinate dehydrogenase, which are closely related enzymes (van Hellemond and Tielens, 1994). The thiol:fumarate reductase for *M. thermoautotrophicum* ΔH (MT1850) (Heim *et al.*, 1998) has not been found to catalyze the reduction of a mixture of the 1,3,4,5-pentenetetracarboxylic acid isomers (R. Hedderich, personal communication, 1999). We propose a single enzyme for steps 7 and 8, which would catalyze the addition of hydrogen to the C-3 *re* face of either **LXV** or **LXVII** substrates. In this manner, the absolute stereochemistry of this carbon would be fixed in the required (*S*) configuration for all subsequent intermediates in this pathway (see later discussion).

We propose that **LXVIII** and **LXIX** are each converted to their respective CoA thioesters, **LXX** and **LXXII** (Fig. 7, reactions 9 and 10), followed by reductive carboxylation to 1-keto-1,3,4,6-hexanetetracarboxylic acid (KHTCA) isomers **LXXIII** and **LXXIV** (Fig. 7, reactions 11 and 12). Reaction 9 has been confirmed by the demonstration that **LXVIII** is converted to both **LXXIII** and **LXXIV** when incubated with HSCoA, HCO_3^-, and ATP. The detection of both **LXXIII** and **LXXIV**, which would result from epimerization of one or more of the asymmetric centers of the KHTCA molecule, was unexpected. The reason for this epimerization has been traced to the rapid exchange of the C-3 hydrogens of KHTCA with the protons of water. This exchange of the C-3 hydrogens of KHTCA is explained by the formation of the enol of the keto acid, which generates a vinylogous analog of malonic acid and as a result would be expected to have acidic methylene hydrogens leading to epimerization at C-3.

The formation of the CoA thioester of **LXVII** and **LXIX** required for steps 9 and 10 (and possibly also for steps 15 and 16 discussed later) could be carried out by several different routes that have been identified in the archaea. These routes could involve the sequential action of enzymes related to acetate kinase and phosphotransacetylase (Latimer and Ferry, 1993), an enzyme related to acetyl-CoA synthase (ADP-forming) (EC 6.2.1.1.3) (Glasemacher *et al.*, 1997), an enzyme related to the coenzyme A-transferases (Jacob *et al.*, 1997), or more likely an enzyme related to acetyl-CoA synthase (Eggen *et al.*, 1991; Jetten *et al.*, 1989).

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The exact route used for the formation of the coenzyme A thioesters in HTCA biosynthesis in the methanoarchaea has yet to be established.

The reductive carboxylation of acyl-CoA thioesters to α-ketoacids is a well-characterized reaction in the archaea. This reaction is known to be catalyzed by α-ketoacid ferredoxin oxidoreductases (α-ketoacid oxidoreductases) (Bock *et al.*, 1996; Heider *et al.*, 1996; Kletzin and Adams, 1996; Tersteegen *et al.*, 1997; Yoon *et al.*, 1996), which are not restricted to the archaea (Gehring and Arnon, 1972). These enzymes catalyze the reversible conversion of acyl-CoA thioesters and CO_2 to α-ketoacids and HSCoA in the presence of low-potential electron donors/acceptors. These enzymes contain thiamine pyrophosphate (TPP) and Fe/S centers and appear to proceed by a radical mechanism, as shown in Fig. 8 (Menon and Ragsdale, 1997). The largest group of α-ketoacid oxidoreductases have a tetrameric αβγδ structure, but through gene fusions of the subunit genes, enzymes with only one, two, and three subunits have also been identified (Bock *et al.*, 1996; Kletzin and Adams, 1996; Tersteegen *et al.*, 1997). Several sets of genes for this type of enzyme are present in *M. jannaschii*, *M. thermoautotrophicum* ΔH, and *A. fulgidus* genomes, one set of which is likely be involved in catalyzing the required carboxylation of **LXVIII** and **LXIX** in the biosynthesis of KHTCA.

Incubation of a cell-free extract of *M. thermophila* with a mixture of the KHTCA isomers, NADH, and NADPH produces about equal amounts of the *arabino*- and *lyxo*-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid (HHTCA) diastereomers. This reduction of the KHTCA isomers (Fig. 7, reactions 13 and 14) could proceed either by a NADH- or a NADPH-dependent reduction, such as occurs with the pyridine nucleotide-dependent 2-hydroxyacid dehydrogenases such as lactate or malate dehydrogenase (Kim and Whitesides, 1988), or by a non-pyridine nucleotide-dependent 2-ketoacid reductase (Neumann and Simon, 1984; Simon *et al.*, 1985). Currently nothing is known about the genes for the non-pyridine nucleotide-dependent 2-ketoacid reductase. Two malate dehydrogenases (Mdh), designated Mdh I and Mdh II, however, have been isolated from *M. thermoautotrophicum* strain Marburg and, based on their N-terminal sequences, have most likely been encoded from the *M. thermoautotrophicum* strain ΔH genes MT1205 and MT0188, respectively (Thompson *et al.*, 1998).

We have cloned and overexpressed the proteins derived from the *M. jannaschii* genes MJ1425 and MJ0490 and have demonstrated that they catalyze the NADH/NADPH reduction of a series of different α-ketoacids to (*S*)-2-hydroxyacids. The enzyme from MJ1425, Mdh I, has been found to reduce the equilibrium mixture of the KHTCA isomers to only the *xylo* isomer of HHTCA (**LXXI**). On the basis of the stereospecificity of

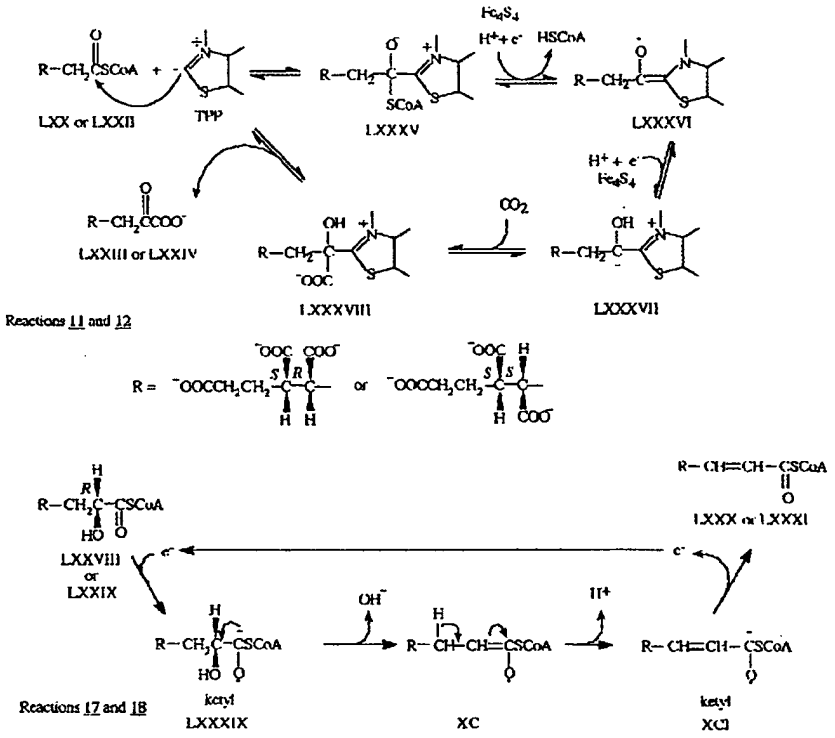
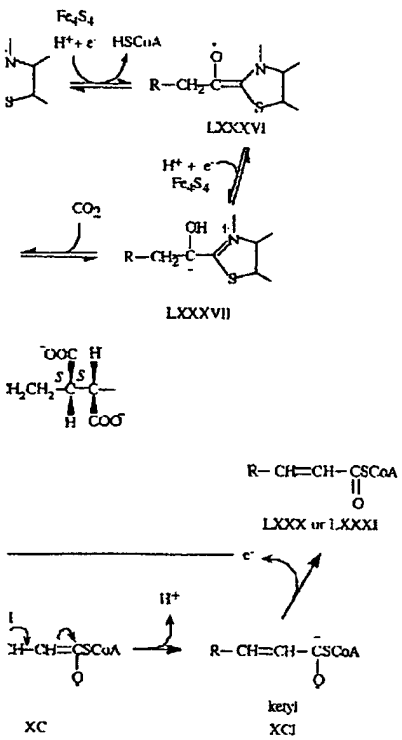


FIG. 8. Reaction mechanisms for reactions 11 and 12 (top) and 17 and 18 (bottom) in the biosynthesis of the 1,3,4,6-hexanetetracarboxylic acid moiety in methanofuran (see Fig. 7). TPP, Thiamine pyrophosphate; **LXX**, coenzyme A ester of L-*erythro*-(2*R*,3*S*)-1,2,3,5-pentanetetracarboxylic acid; **LXXII**, coenzyme A ester of L-*threo*-(2*S*,3*S*)-1,2,3,5-pentanetetracarboxylic acid; **LXXVIII**, coenzyme A ester of (1*R*,3*R*,4*S*)-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid; **LXXIX**, coenzyme A ester of (1*S*,3*R*,4*S*)-1-hydroxy-1,3,4,6-hexanetetracarboxylic acid; **LXXXV**, anion of hemithioacetal intermediate; **LXXXVI**, hydroxyethyl-TPP radical intermediate; **LXXXVII**, carbanion of hydroxyethyl-TPP intermediate; **LXXXVIII**, hydroxyethyl-TPP intermediate; **LXXXIX** and **XCI**, ketyl radical intermediates; **XC**, enoxy radical intermediate.

this enzyme, this isomer is assigned as L-*xylo*-HHTCA (**LXXI**) (see Fig. 7 for the absolute stereochemistry and structure of these compounds). Since this isomer has not been found to be an intermediate in HTCA biosynthesis, whereas DL-*arabino*-HHTCA has been found to be an intermediate, it is concluded that L-*arabino*-HHTCA (**LXXV**) and L-*lyxo*-HHTCA (**LXXVII**) are the true intermediates in the pathway. Since



11 and 12 (top) and 17 and 18 (bottom) in boxylic acid moiety in methanofuran (see , coenzyme A ester of L-*erythro*-(2*R*,3*S*)- enzyme A ester of L-*threo*-(2*S*,3*S*)-1,2,3,5- nzyme A ester of (1*R*,3*R*,4*S*)-1-hydroxy- enzyme A ester of (1*S*,3*R*,4*S*)-1-hydroxy-1,) of hemithioacetal intermediate; **LXXXVI, XVII**, carbanion of hydroxyethyl-TPP in- ermediate; **LXXXIX** and **XCI**, ketyl radi- iate.

is L-*xylo*-HHTCA (**LXXI**) (see Fig. id structure of these compounds). l to be an intermediate in HTCA HTCA has been found to be an in- *bino*-HHTCA (**LXXV**) and L-*lyxo*- rmediates in the pathway. Since

there is no mechanism for the stereochemistry at C-3 to change in any intermediate in the pathway after **LXV, LXVI,** and **LXVII,** the assignment of the absolute stereochemistry can be made to all of the intermediates, as indicated in Fig. 7.

The final steps in the pathway from **LXXV** or **LXXVII** to HTCA could involve the reverse elimination of water from these molecules to form L-*erythro*-(*E* or *Z*)-1,3,5,6-hex-1-enetetracarboxylic acid and L-*threo*-(*E* or *Z*)-1,3,5,6-hex-1-enetetracarboxylic acid, respectively. After equilibration of these two isomers by racemization at C-3, and specific reduction of the L-*erythro*-(*E* or *Z*)-1-HETCA, the final product HTCA would be produced. The occurrence of this racemization is confirmed by the rapid exchange of the C-3 hydrogens of *trans*-glutaconic acid and is a required step in the biosynthetic pathway because of the *meso* stereochemistry of the final HTCA (White *et al.,* 1996). This pathway is consistent with the experimental observation that incubation of cell extracts with DL-*arabino*-HHTCA leads to the production of *erythro*-(*E* or *Z*)-1-HETCA and HTCA.

Although this is a possible route for the final steps in the biosynthetic scheme, the experimental evidence would suggest that these last three reactions in fact occur with the coenzyme A derivative of **LXXV** and **LXXVII.** The experimental data show that the conversion of DL-*arabino*-HHTCA to *erythro*-(*E*)-1-HETCA and HTCA is stimulated by the addition of HSCoA and ATP to the incubation mixture. This reaction would be catalyzed by an enzyme(s) similar to the (*R*)-lactyl-CoA desaturase involved in the acrylic acid pathway of lactate metabolism (Kuchta and Abeles, 1985; Kuchta *et al.,*1986; Hofmeister and Buckel, 1992) or to the (*R*)-2-hydroxyglutaryl-CoA dehydratase involved in the fermentation of glutamate via the hydroxyglutarate pathway (Klees *et al.,* 1992; Bendart *et al.,* 1993). All of these enzymes are oxygen sensitive, they all likely contain Fe/S centers, and they all catalyze the elimination of 2-hydroxyacids with the same (*R*) stereochemistry found in our intermediates. On the basis of the work in Buckel's laboratory (Buckel and Golding, 1999), reactions 17 and 18 appear to proceed by a radical mechanism with ketyl intermediates, as shown in Fig. 8. The *M. jannaschii* genome contains a gene for 2-hydroxyglutaryl-CoA dehydratase (MJ0007) and two genes for the activator protein of (*R*)-2-hydroxyglutaryl-CoA dehydratase (MJ0004 and MJ0800). An open reading frame predicted to code for the activator protein of (*R*)-2-hydroxyglutaryl-CoA dehydratase and the β subunit of (*R*)-2-hydroxyglutaryl-CoA dehydratase has also been identified in *Methanopyrus kandeleri* (Vorholt *et al.,* 1997). We have now confirmed that the enzymes generated from the protein products of the *M. jannaschii* genes MJ0004 and MJ0007 function as a (*R*)-2-hydroxyglutarylCoA dehydratase and

are involved in the biosynthesis of β-glutamate. We thus propose that an enzyme composed of the gene products of both the MJ0007 and MJ0800 genes is involved in performing the same type of reaction on the intermediates **LXXVIII** and **LXXIX.**

Additional support for the involvement of the CoA ester in the final steps of HTCA biosynthesis comes from the fact that most of the known examples of the reduction of α,β-unsaturated acids occur as their CoA esters (Caldeira *et al.,* 1996; Sedlmeier and Simon, 1985; Weeks and Wakil, 1969). One advantage of using a CoA ester in these last steps of the reaction is that the carboxylate group of HTCA is activated at the correct position for its coupling to the α-amino group of glutamic acid in the biosynthesis of methanofuran.

In summary, we find that all of the reactions used in the construction of the HTCA molecule are analogous to reactions previously described in bacterial metabolism, and each reaction is likely catalyzed by paralogs of the enzymes catalyzing these reactions. This finding is analogous to that observed in the biosynthesis of coenzyme B.

V. Biosynthesis of Coenzyme F_{420}

Little work has been published on the biosynthesis of coenzyme F_{420}, a hydride transfer coenzyme that functions in a manner similar to NADH (Jacobson and Walsh, 1984). The most recent work published on F_{420} biosynthesis is from Bacher's laboratory, where it has been demonstrated that growing cells of *M. thermoautotrophicum* readily incorporate 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione (**XLIII**) (Fig. 9) and 4-hydroxyphenylpyruvate (**XLVI**) into the 7,8-didemethyl-8-hydroxy-5-deazariboflavin moiety (**LI**) of coenzyme F_{420} (**CVIII**) (Fo) (Reuke *et al.,* 1992). Compound **XLIII,** an established intermediate in riboflavin biosynthesis (Bacher *et al.,* 1996; Richter *et al.,* 1997), has been proposed to be formed by the same route as that found in bacterial riboflavin biosynthesis, and thus it represents the last common intermediate in the biosynthesis of riboflavin and Fo. The bacterial pathway, however, cannot be completely identical to the proposed riboflavin biosynthesis pathway in archaea since the methanoarchaea appear to lack GTP cyclohydrolase II, the first enzyme in riboflavin biosynthesis (Howell and White, 1997). Our current ideas for the pathway are outlined in Fig. 9, which shows a possible route for the formation of Fo (**LI**) from GTP, and in Fig. 10, which shows a possible route for the conversion of **LI** to coenzyme F_{420}. We have established, using incubations with cell extracts of *M. thermophila,* that **XLIII** and **XLVI** (Fig. 9) are the precursors for the formation of the Fo moiety of F_{420} (**LI**), and that

-glutamate. We thus propose that roducts of both the MJ0007 and ning the same type of reaction on XIX.

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Coenzyme F_{420}

the biosynthesis of coenzyme F_{420}, unctions in a manner similar to The most recent work published on oratory, where it has been demon- *moautotrophicum* readily incorpo- 3*H*)-pyrimidinedione (**XLIII**) (Fig. VI) into the 7,8-didemethyl-8-hy-) of coenzyme F_{420} (**CVIII**) (Fo) **II**, an established intermediate in *L.*, 1996; Richter *et al.*, 1997), has ame route as that found in bacter- it represents the last common in- oflavin and Fo. The bacterial path- dentical to the proposed riboflavin ce the methanoarchaea appear to enzyme in riboflavin biosynthesis ent ideas for the pathway are out- e route for the formation of Fo (**LI**) ws a possible route for the conver- ve established, using incubations that **XLIII** and **XLVI** (Fig. 9) are ie Fo moiety of F_{420} (**LI**), and that

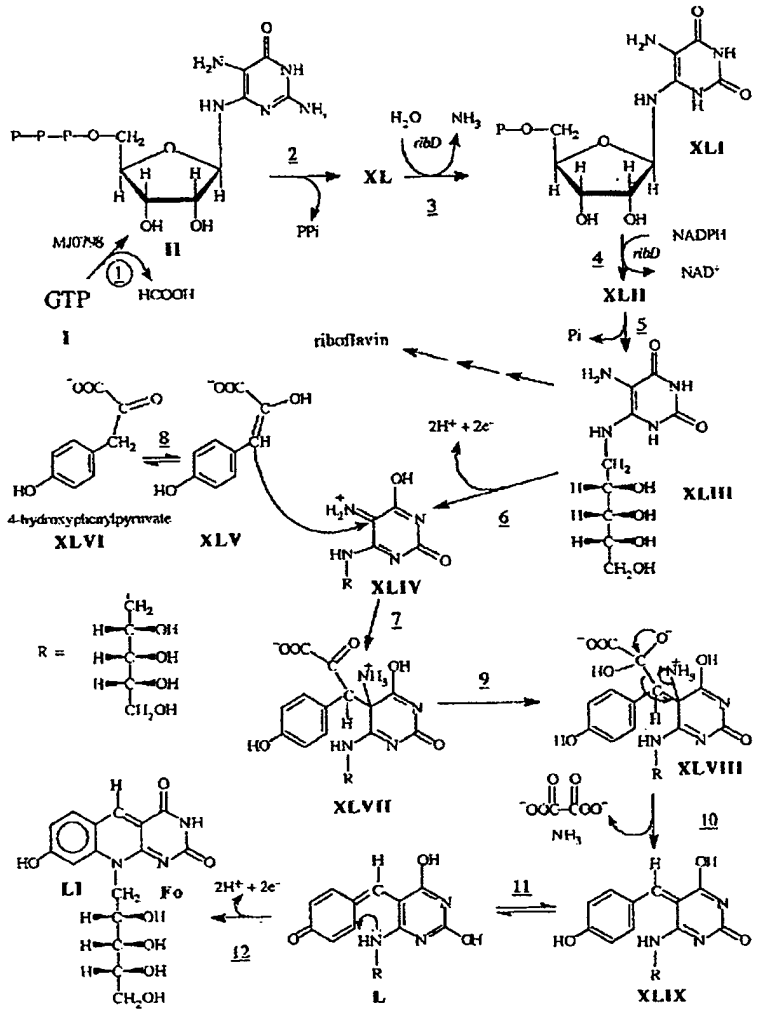

FIG. 9. Proposed pathway for the biosynthesis of the Fo moiety of coenzyme F_{420}. **I**, Guanosine triphosphate; **II**, triaminopyrimidine triphosphate; **XL**, 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate; **XLI**, 2,5-diamino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate; **XLII**, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate; **XLIII**, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; **XLIV**, oxidized **XLIII**; **XLV**, 4-hydroxyphenylpyruvate; **XLVI**, enol form of 4-hydroxyphenylpyruvate; **XLVII**, condensation product between **XLIV** and **XLVI**; **XLVIII**, **XLIX**, and **L**, intermediates in the formation of Fo; **LI**, Fo, 7,8-didemethyl-8-hydroxy-5-deazariboflavin.

lactyl phosphate, formed by the GTP-dependent phosphorylation of lactate (CII), serves as an intermediate in the formation of the phosphodiester bond in F_{420}.

The first reaction in the methanogens is catalyzed by GTP cyclohydrolase III (Fig. 9, reaction 1). We propose that the triaminopyrimidine triphosphate (II) shown in Fig. 9 is the true intermediate in the archaeal pathway. This very reactive compound would therefore serve as the branch point for the biosynthesis of F_{420}, flavin adenine dinucleotide (FAD), methanopterin, and possibly also the 7-deazaguanosine-containing series of bases found in tRNA nucleosides (Watanabe *et al.*, 1997). After removal of pyrophosphate from II (Fig. 9, reaction 2), the resulting **XL** would be transformed to **XLIII** by the same series of reactions as occurs in riboflavin biosynthesis. Possible genes in the genome of *M. jannaschii* have been proposed to encode enzymes that catalyze the two reactions in the formation of **XLIII** (Koonin *et al.*, 1997). These are MJ0430 or MJ1102 for the *ribD* pyrimidine deaminase domain (Fig. 9, reaction 3) and MJ0671 for the *ribD* pyrimidine reductase domain (Fig. 9, reaction 4). We have now confirmed that MJ0671 is the gene encoding for the pyrimidine reductase and that the gene product of MJ0430 is a dCTP deaminase.

Compound **XLIII**, after a two-electron oxidation to compound **XLIV**, condenses with **XLVI**, likely as the enol **XLV** (Fig. 9, reaction 7), to form compound **XLVII**, which, after the series of indicated reactions, leads to the formation of reduced Fo. A proposed series of reactions is shown in Fig. 9. Reduced Fo would be readily oxidized to Fo or could proceed down the pathway as the reduced form of Fo (H_2Fo). The involvement of oxalate in the biosynthesis of Fo is supported by our demonstration of the presence of low concentrations of oxalate in cell extracts of *M. thermoautotrophicum* and our demonstration of oxalate production in a cell extract of *M. jannaschii* incubated with **XLVI** and **XLIII**. Predictions as to the identity of the enzyme(s) involved in the biosynthesis based on sequence comparisons are currently impossible since any analogous reactions for comparison are unknown. We considered it very likely that a single enzyme could catalyze reactions 7 to 11 in Fig. 9 based on similar complex transformations carried out by such enzymes as GTP cyclohydrolase I (Nar *et al.*, 1995) and 6,7-dimethyl-8-ribityllumazine synthase in riboflavin biosynthesis (Bacher *et al.*, 1996). This has now been confirmed, in our laboratory, for an enzyme cloned from *M. jannaschii*.

We have also studied the formation of the phosphodiester bond in the coenzyme F_{420} structure and find that it is readily formed when cell extracts of *M. thermophila* or *M. jannaschii* are incubated with Fo (LI), GTP (I), and L-lactate (CII) (Fig. 10). The formation of this bond is great-

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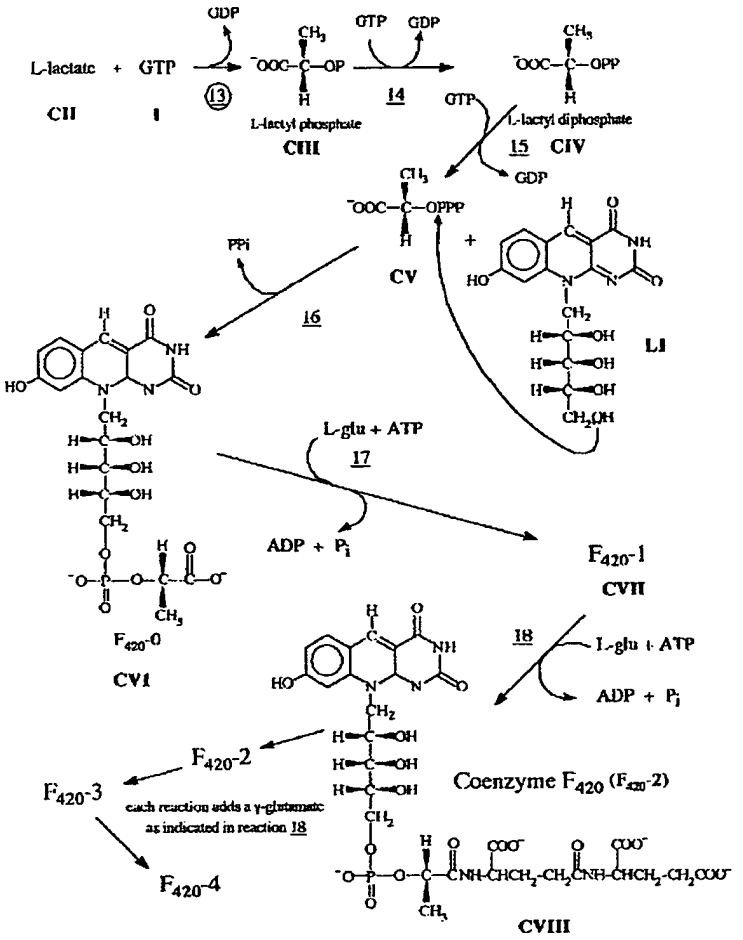

FIG. 10. Proposed pathway for the biosynthesis of coenzyme F_{420} from **LI**. **LI**, Fo, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; **CII**, L-lactate; **CIII**, L-lactyl phosphate; **CIV**, L-lactyl diphosphate; **CV**, L-lactyl triphosphate; **CVI**, F_{420}–0; **CVII**, F_{420}–1; **CVIII**, F_{420}–2, coenzyme F_{420}.

ly stimulated by including in the incubation mixture an unknown prod-uct obtained by treating L-lactic acid with $POCl_3$. At present, we propose the pathway shown in Fig. 10 as a possible pathway for the generation of F_{420} (F_{420}–2) and its γ-glutamyl derivatives (F_{420}–1, F_{420}–3, F_{420}–4,

etc). The first step in this pathway would begin with the GTP-dependent phosphorylation of L-lactate (**CII**) to L-lactyl phosphate (**CIII**). The resulting **CIII** is then phosphorylated by GTP in two consecutive reactions, first to L-lactyldiphosphate (**CIV**) and then finally to L-lactyl triphosphate (**CV**). This series of reactions is the same that is used in nature in the biosynthesis of the phosphodiesters in nucleic acids. The nature of the compound formed from treating L-lactic acid with $POCl_3$, which stimulates **CVI** synthesis, has not as yet been identified, but it is known not to be **CIV** or **CV**. The current evidence indicates that it is a cyclic derivative of **CIV** that undergoes a ring opening to form **CIV**, which then serves as a precursor to **CVI**, as shown in Fig. 10.

We have confirmed the presence of lactoyl phosphate in *M. thermophila* and *M. thermoautotrophicum* ΔH by gas chromatography–mass spectrometry (GC-MS) analysis. We have also shown that cell extracts of *M. thermophila* incubated with [*hydroxy*-^{18}O]lactate and GTP produce ^{18}O-labeled **CIII**.

Another important aspect of the coenzyme F_{420} structure is the presence of γ-linked glutamic acid residues. This structural element is also a common characteristic of some of the different methanofurans (Daniels, 1993) and folates, and it identifies another common structural element uniting these coenzymes, as does the pyrophosphate diester linkage present in NAD, FAD, coenzyme A, and molybdopterin. The mechanism and enzymology for the assembly of the γ-linked glutamic acid have been studied in bacteria and in eukaryotes, where the reaction is involved in the generation of the folyl-γ-polyglutamates (Banerjee *et al.*, 1988; Garrow *et al.*, 1992).

The enzymes responsible for these reactions are the folyl-γ-polyglutamate synthases (EC 6.3.2.17), and they have been found to be moderately conserved between bacteria and eukaryotes. A paper on the X-ray structure of the *Lactobacillus casei* enzyme reveals that the enzyme is a modular protein consisting of two domains, one with a typical mononucleotide-binding fold and the other strikingly like that in the folate-binding enzyme dihydrofolate reductase (Sun *et al.*, 1998). If the archaea contained an enzyme(s) related to this enzyme, we would expect at least a portion of the enzyme sequence to have some homology to the folylpolyglutamate synthases. However, no open reading frames in the genomes of *M. jannaschii* or *M. thermoautotrophicum* ΔH can be found that have sequences homologous to any known folylpolyglutamate synthase. We have three possible explanations for this finding: (1) the sequences of the archaeal enzymes have diverged to such an extent from the known enzymes that it is impossible to detect them by sequence homologies, (2) a nonortholog replacement for these enzymes

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impossible to detect them by se-
; replacement for these enzymes

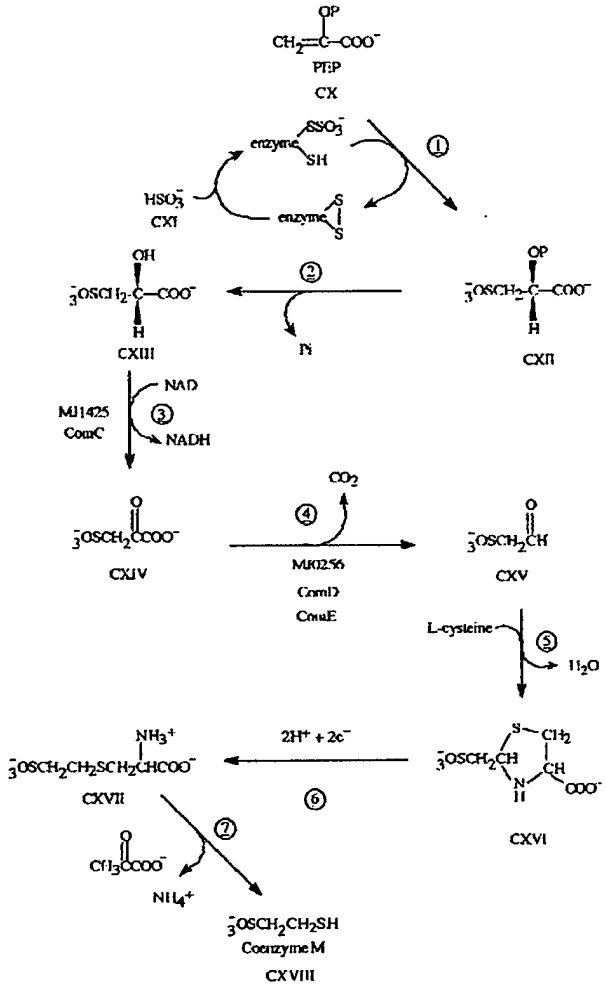

FIG. 11. Proposed pathway for the biosynthesis of coenzyme M. **CX**, Phosphoenol pyruvate; **CXI**, bisulfite; **CXII**, L-sulfolactyl phosphate; **CXIII**, L-sulfolactate; **CXIV**, sulfopyruvate; **CXV**, sulfoacetaldehyde; **CXVI**, 2-(sulfomethyl)thiazolidine-4-carboxylic acid; **CXVII**, sulfoethylcysteine; **CXVIII**, coenzyme M, 2-mercaptoethanesulfonic acid.

exists in the archaea, or (3) the peptide bond-forming reaction proceeds by another mechanism and would thus be expected to use a different enzyme. We have also identified an α-glutamyl-linked F_{420} in *M. jannaschii* that further establishes biochemical similarities between bacterial metabolism, which also produces α-glutamyl-linked folates (Ferone *et al.*, 1986), and the metabolism found in the Archaea.

VI. Biosynthesis of Coenzyme M

The currently established pathway for the biosynthesis of coenzyme M is outlined in Fig. 11. We have established that reaction 3 is carried out by the gene product of the *M. jannaschii* gene MJ1425 (Graupner and White, 2000). The TPP-dependent decarboxylation of reaction 4 is carried out by the enzyme composed from the two proteins produced by the MJ0256 gene, which are designated as ComD and ComE. These correspond to the MTH1206 and MTH1207 genes in *M. thermoautotrophicum* ΔH. The ComD and ComE proteins make an $\alpha_6\beta_6$ dodecamer enzyme that is a member of the acetolactate and pyruvate oxidase family of enzymes (Chang, 1992). Both subunits align with different portions of the phosphopyruvate decarboxylase (Schwartz *et al.*, 1998), an enzyme involved in the biosynthesis of compounds containing a P—C bond (Hidaka *et al.*, 1995).

We are currently trying to establish the identity of the other genes in the pathway, each of which has an analogous reaction occurring in other known enzymatic reactions. These include formation of the sulfonic acid derivative by a route analogous to that involved in the biosynthesis of UDP-sulfoquinovose, an intermediate in the biosynthesis of sulfolipids (Fig. 11, reaction 1) (Benning, 1998); the Stickland reaction used for reduction of the C—N bond in the thiazolidine intermediate (reaction 5) that occurs in D-proline reductase (Kabisch *et al.*, 1999); and a β-elimination of pyruvate and ammonia from sulfocthylcysteine (reaction 7) by a reaction like that catalyzed by β-cystathionase (Flavin and Slaughter, 1964).

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